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A Model of Drug Delivery to Normal and Cancer Cells by Antibody-Targeted Nanoliposomes

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A mathematical model of drug delivery to cells by antibody-conjugated liposomes is presented. The model includes variables describing concentration of free and liposome-encapsulated drug in medium, intracellular drug amount and concentration of target cells. It accounts for mechanisms of active and passive transport through cell membrane and Hill-type dependence of rate of cell death on intracellular drug amount. The model has been applied to data for cytotoxic effect of free or liposome-encapsulated doxorubicin on target melanoma cells in culture. Fitting of experimental data showed increased efficacy of 24 hours exposure with both free and liposome encapsulated doxorubicin. Simulation with parameter values after data fitting demonstrated the existence of temporary increase in intracellular amount of toxin per cell with liposome delivery, more efficient cell killing of tumor cells by liposome-encapsulated drug in culture consisting of mixture of tumor and normal cells, and non-linear dependence of therapeutic effect on drug concentration for normal and tumor cell mixture. The results may help in the design of therapeutic and diagnostic approaches.

Keywords: Nanoliposome, Drug Delivery, Cell Killing, Cancer.

1. INTRODUCTION

Drugs, toxins, and their derivatives as antibody-toxin fusion proteins have shown anti-tumor activity in humans. However, their efficacy is limited by non-specific toxicity caused by binding and killing of normal cells. Encapsulation of drugs or toxins in liposomes could decrease the extent of nonspecific toxicity if the uptake of liposome-encapsulated molecules to cancer cells is more efficient than the uptake of free molecules (see for review Refs. [1–3]). Major differences between uptake of liposome-encapsulated and free molecules relate to half-life, penetration, avidity and capacity as a ratio of toxin to targeting molecules. In an attempt to quantify the contribution of these factors we developed a model describing drug delivery to cells by drug-encapsulating antibody-conjugated liposomes.

Conventional liposomes consisting of naturally occurring phospholipids and cholesterol are rapidly removed from circulation by the liver, spleen, and the other parts of reticular-endothelial system after *in vivo* administration.[4] Long circulating drug-encapsulating liposomes have increased drug-carrying properties and were shown to be effective against many types of tumors.[5–10] Schematic representation of potential pathways to achieve intracellular release for free or liposome-entrapped drug is shown in Figure 1. After entering the blood liposomes can be transported to the extracellular space and bind specifically (targeted delivery) or nonspecifically to cells.[11,12] Antibody molecules or fusion peptides conjugated to the surface of liposome can provide binding with subsequent internalization of liposome into cell or with fusion of liposome to the cell membrane. Fusion leads to formation of pores connecting the interior of the liposomes with the target cell cytoplasm and subsequent intracellular drug release. After internalization, liposome undertakes intracellular destruction with the following delivery of drug.[13–16] In the extracellular space liposomes release free drug molecules due to the instability of their structure. Released drug molecules can be internalized into cells and initiate cascade of intracellular reactions leading to the cell death. They can also diffuse nonspecifically over the cell membrane although, in general, this route is not an option for macromolecules. For some drugs both extracellular and intracellular mechanisms of cell killing can coexist inducing apoptosis or cell necrosis.[17] Alternative mechanism of direct cytosolic delivery of macromolecules is described in Ref. [18] when cell-penetrating peptides can provide delivering various cargos including liposomes.[19–21]

Various mathematical models of different complexity (having from 1 up to 12 parameters) were developed to analyze dependence of cytotoxic effect of drugs on drug concentration.[22–30] Comparative analysis of some of these

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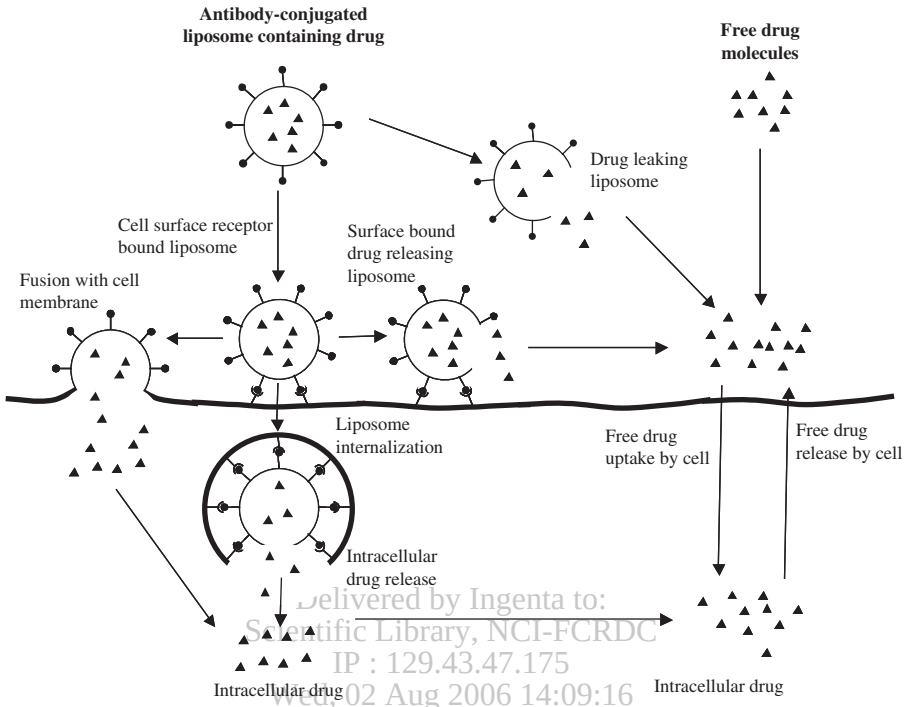


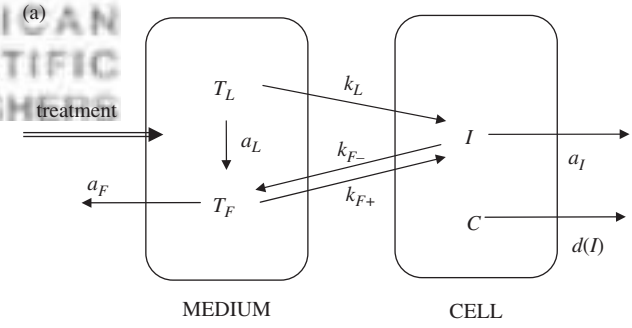
Fig. 1. Schematic representation of potential pathways for delivery of free or liposome-encapsulated drug. Liposome conjugated with molecules specific to the receptors on target cell surface can bind to the cell and then fuse to the cell membrane or release drug into extracellular space. Cells can uptake liposome by internalization with subsequent intracellular release of encapsulated drug. Free drug molecules can diffuse over the plasma membrane.

models was described in Ref. [24] where models were fit to cytotoxicity data for doxorubicin acting on various cancer cell lines. The data analysis showed that two mechanisms peak concentration model[24] provides the best fit in all but two cases when more simple models demonstrated better or comparable fitting.

In the present work we built a model that includes dynamics of concentration of free and liposome-encapsulated drug in medium, intracellular drug amount and concentration of target cells. The model describes equilibration of intracellular and extracellular concentrations of drug by first-order kinetics mechanism of active or passive transport through cell membrane, drug uptake by second-order kinetic depending on extracellular drug and cell concentration, and cell killing by Hill-type dependence of rate of cell death on intracellular drug amount.

2. THEORETICAL MODEL

Two-compartmental kinetic model of drug-encapsulating antibody-conjugated liposomes and free molecules interaction with cells is represented in the Figures 2a, b. Toxin (drug) molecules are introduced either in toxin-encapsulated liposomes T_L or as free molecules T_F. Elimination of free molecules from medium occurs with rate a_F. Liposome destruction leads to the release of free molecules with rate a_L. Cells C uptake liposome-encapsulated drug and free molecules with rates k_L and k_{F+}, respectively, and this leads to the increase of total amount of intracellular drug I. Cells release only free molecules due to the passive or active transport with the rate k_{F-}, which, in general, is different from k_{F+}. Cells can also grow with the rate g and die with the normal rate d_0 in the absence of intracellular



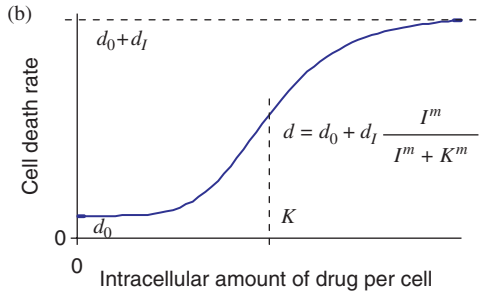


Fig. 2. (a) Kinetic model of toxin delivery to cells. (b) Dependence of target cell death on intracellular amount of drug.

drug. We assume that death rate increases with the increase of intracellular drug amount I and can reach maximum of $d_0 + d_I$ for high enough values of I.

This two-compartmental model can be described by system of differential equations. Dynamics of liposome-encapsulated drug concentration T_L can be written as:

$$\frac{dT_L}{dt} = -k_L T_L C - a_L T_L \quad (1)$$

where a_L is the rate of liposome destruction in medium (or natural leaking due to liposome instability) and $k_L T_L C$ describes liposome-encapsulated drug uptake by cells. Here we do not describe in details different ways of drug deliver to the cells. It should be noted also that T_L corresponds to the average liposome-encapsulated drug concentration in medium, not the concentration of liposomes.

The equation for free molecules T_F is the following:

$$\frac{dT_F}{dt} = -k_{F+} T_F C + k_{F-} IC/V_C + a_L T_L - a_F T_F \quad (2)$$

where a_L, rate of liposome leaking leading to the release of free drug molecules. Because T_L corresponds to the average concentration of liposome-encapsulated drug in medium we use the same parameter a_L for equations describing dynamics of T_L and T_F. The last two terms ($k_{F+} T_F C$, $k_{F-} IC/V_C$) describe free drug molecules uptake and release by cells; V_C, cell volume.

Intracellular drug amount per cell I in this model is described as:

$$\frac{dI}{dt} = k_L T_L + k_{F+} T_F - k_{F-} I/V_C - a_I I \quad (3)$$

Here $k_L T_L$ and $k_{F+} T_F$ are the rates of uptake of free and liposome-encapsulated drug by cell; a_I, rate of intracellular drug destruction; V_C, as it was described above, cell volume. Release of free drug molecules is described by $k_{F-} I/V_C$.

The last equation of the system shows cell dynamics:

$$\frac{dC}{dt} = gC - dC \quad (4)$$

where g and d, are the rates of cell growth and death, respectively. Here we assume that death rate d depends on normal cell death rate d_0 and concentration of intracellular drug molecules per cell (I):

$$d = d_0 + d_I \frac{I^m}{I^m + K^m} \quad (5)$$

where d_I is the maximum increase in rate of cell death; K, amount of intracellular drug per cell giving 50% of maximum increase in death rate; and m, slope parameter. So, in the absence of drug in medium, intracellular amount of drug per cells I is equal to 0 and death rate for cells is equal to its normal value d_0. After treatment, with the increase of intracellular amount of drug per cell death rate increases and can reach maximum value of $d_0 + d_I$ when intracellular amount of drug per cell is large enough.

Finally, Eqs. (1–5) describes the kinetic model of interaction of liposome-encapsulated drug and free drug molecules with cells. Initial conditions for this system corresponding to the absence of treatment can be described as:

$$T_L(0) = 0, \quad T_F(0) = 0, \quad I(0) = 0, \quad C(0) = C_0 \quad (6)$$

Treatment with liposome-encapsulated or free drug can be defined for the model by changing the initial values of the variables T_L and T_F.

If one assumes that cell concentration is constant, and drug molecules are stable both inside and outside cell ($a_L = a_F = a_I = 0$), it is easy to show that system reaches equilibrium at $T_F = I/V_C$, which means that concentrations of free drug inside and outside cells are equal.

For all simulation experiments and data fitting, model solutions were calculated numerically. In data fitting non-weighted sum of square deviations of the predicted values from data points was minimized.

3. *IN VITRO* EXPERIMENT MODELING

The model described above was used for fitting data presented in Ref. [23]. Model variables: Concentration of doxorubicin (DOX) (free or encapsulated in liposomes, μg/ml); concentration of cells (cells/ml) and the intracellular DOX amount per cell (μg/cell). To model dynamics of control (no treatment) cells N(cells/ml) the following equation was added to the system (1–5):

$$\frac{dN}{dt} = gN - d_0 N \quad (7)$$

where cells have constant death rate d_0. So, survival relative to control can be calculated as C/N 100%.

As described in Ref. [23] B16F10 cells (1.6×10^5 cells/ml) were treated with doxorubicin and DOX uptake was measured after 3 hours of exposure with 5 different concentrations of free and liposome-encapsulated toxin. Using the data from Ref. [23] we estimated parameters $k_L = 3.09 \times 10^{-7}$ ml$\cdot$ cells$^{-1}\cdot$ hour^{-1} and $k_{F+} = 1.08 \times 10^{-7}$ ml/cells/hour (here we assumed that there is no free intracellular DOX efflux from cell, $k_{F-} = 0$). These values can provide uptake of femtomoles of DOX per cell (from 0.6 to 32 fmole/cell) after 3 hours in presence of 0.5–20 μM of DOX in medium as reported by Eliaz et al.

Rate constant of B16F10 cells proliferation estimated in this work is $g = 0.030$ hour^{-1}. Let us assume that rate of natural cell death is close to the rate of proliferation: $d_0 = 0.029$ hour^{-1}. For simulations we assumed that there is no liposome leakage and toxin molecules are stable inside the cells and in the medium ($a_L = a_F = a_I = 0$). Cell volume can be estimated as 10^{-8} ml assuming that cell radius is about 10–15 μm. All model parameter values are represented in Table I. Values of d_I (hour^{-1}) and K (fg) and m (dimensionless) were specified by data fitting.

Figure 3 shows the results of data fitting for the experiments represented in Ref. [23] with the parameter values

Table I. Nomenclature and parameter values.

	Description	Dimension	Value	Ref.
Variables				
T_L	Liposome-encapsulated drug concentration	μg/ml		
T_F	Free drug concentration	μg/ml		
I	Total intracellular drug	μg/cell		
C	Cells concentration	cell/ml		
Parameters				
a_L	Rate of release of liposome-encapsulated toxin	hour^{-1}	0–0.06	[25]
a_F	Rate of free drug destruction	hour^{-1}	0–0.06	[25]
k_L	Rate of liposome-encapsulated drug uptake by cells	ml cells^{-1} hour^{-1}	3.09×10^{-6}	[23]
k_{F+}	Rate of free drug uptake by cells	ml cells^{-1} hour^{-1}	1.08×10^{-6}	[23]
k_{F-}	Rate of free drug release by cells	ml cells^{-1} hour^{-1}	$0–1.08 \times 10^{-6}$	[23]
g	Rate of cell growth	hour^{-1}	0.030	[23]
d_0	Normal rate of cell death	hour^{-1}	0.029*	
d_I	Maximum increase in death rate for cells	hour^{-1}	~0.050**	
K	Amount of intracellular drug per cell leading to the 50% of maximum increase in death rate	fg	~750**	
m	Slope parameter	—	~1.5**	
V_C	Cell volume	ml	$\sim 10^{-8}$	

*assumption; **average value for the parameter after data fitting.

described above. B16F10 cell were treated with HAL liposomes encapsulating DOX and free DOX molecules. Cytotoxic effect was determined either immediately at 3, 6, 12, 24, 48, 72, and 96 hours after treatment or with the delay at 96 hour irrespective of treatment duration. It was noted in Refs. [23, 31] that there is a lag-time in drug effect with no cell killing for about 3 hours even for very large drug concentration and this effect was not due to the low accumulation of the drug in the cells. This lag-time was accounted by holding cell death rate at value d_0 for the first 3 hours of treatment. Parameter values for each fitting are presented in Table II. For immediate treatment with 3 hours exposure

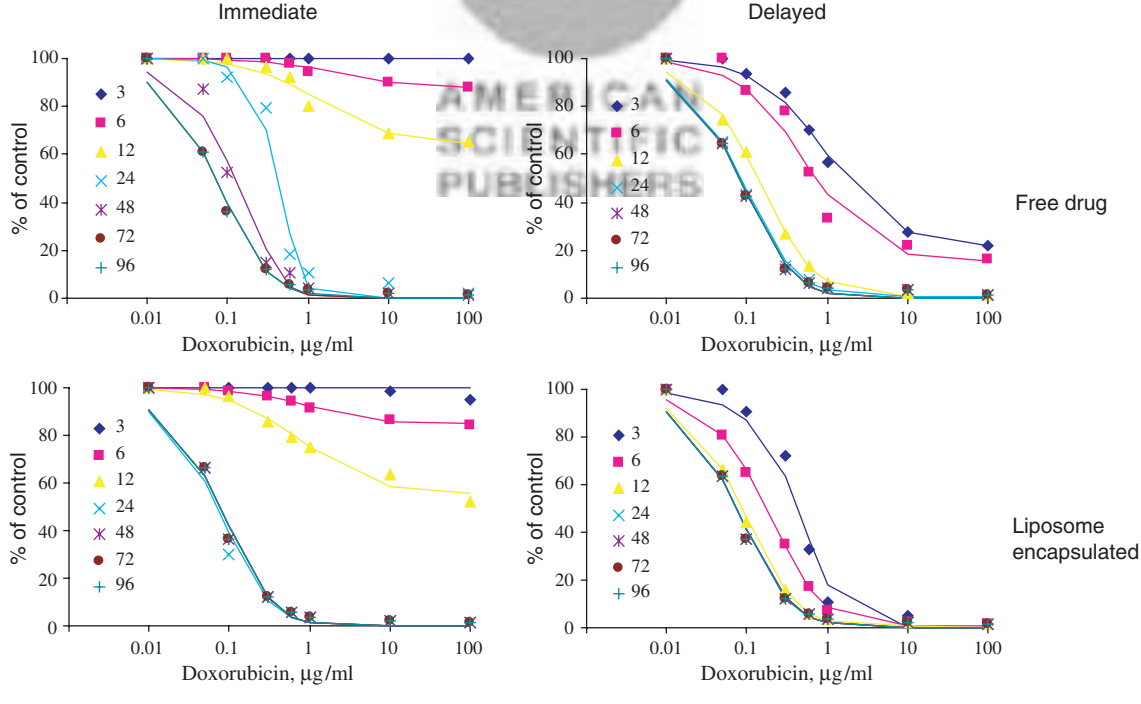


Fig. 3. Model fitting to the data obtained in Ref. [23]: Immediate and delayed cytotoxic effects of exposures for free and liposome encapsulated doxorubicin. Cells were treated with drug for 3, 6, 12, 24, 48, or 96 hours and cytotoxic effect was measured immediately after treatment or at 96 hour independent on treatment duration.

Table II. Parameter values for the data fitting shown in Figure 3.

			Treatment duration for different protocols						
	Delivery	Protocol	3	6	12	24	48	72	96
d_1, hour^{-1}	free	immediate	ND	0.039	0.046	0.181	0.070	0.052	0.038
		delayed	0.016	0.018	0.044	0.037	0.038	0.038	0.038
	liposome	immediate	ND	0.055	0.057	0.153	0.075	0.049	0.036
		delayed	0.041	0.036	0.045	0.041	0.037	0.037	0.037
K, fg	free	immediate	ND	609	823	880	420	489	584
		delayed	506	501	674	433	608	687	706
	liposome	immediate	ND	1158	1058	385	708	825	891
		delayed	858	912	913	914	915	964	972
m, —	free	immediate	ND	1.5	1.7	3.7	2.2	1.5	1.5
		delayed	1.3	1.6	1.1	1.4	1.4	1.4	1.4
	liposome	immediate	ND	1.1	1.3	2.0	1.6	1.6	1.6
		delayed	2.1	1.2	1.2	1.4	1.5	1.5	1.5

*ND = not done.

there were no or small effect in cell killing so values for d_1, m, and K for these cases were not estimated.

It should be noted that parameters values described above can provide (simulations not shown) main features of immediate and delayed treatment: Sigmoidal dose-response; increased effect for prolonged treatment; increase in cell killing with the increase in free or liposome-encapsulated drug concentration; increased effect of delayed treatment comparing to immediate one; and increased effect of treatment for liposome-encapsulated drug comparing to free one. Each dose-dependent curve was fitted separately with all model parameter fixed except d_1 and K and m. Parameter values after treatment are represented in Table II.

4. SIMULATIONS

This model was used for simulation of dynamics of toxin (drug) delivery to cells when free or liposome-encapsulated toxin is used for cell treatment (Fig. 4). Model solutions correspond to the parameters described above. It was assumed for this simulation that cell concentration is constant and only the dynamics of molecules delivery is shown. It was also assumed that free toxin can be passively transferred through membrane inside and outside the cell with equal rates: $k_{F+} = k_{F-}$. Model variables had the following initial values (at $t = 0$): $C_0 = 10^7$ cells/ml; $I_0/V_C = 0$ μg/ml. For treatment with liposome-encapsulated toxin we used values $T_{L0} = 10$ μg/ml, $T_{F0} = 0$; and $T_{F0} = 10$ μg/ml, $T_{L0} = 0$ for treatment with free toxin molecules. One can see in Figure 4 that after 2 hours intracellular and extracellular free toxin molecules concentrations (I/V_C and T_F) reaches equilibrium (for treatment with free toxin this time was about half an hour).

Figure 5 shows simulation for *in vitro* treatment with liposome-encapsulated or free drug for mixture of normal and tumor cells (1:1) with concentration 10^6 cell/ml. It was assumed that tumor cells have 2 times higher rate of uptake of liposome-entrapped toxin ($2 \times k_L$). All other parameters

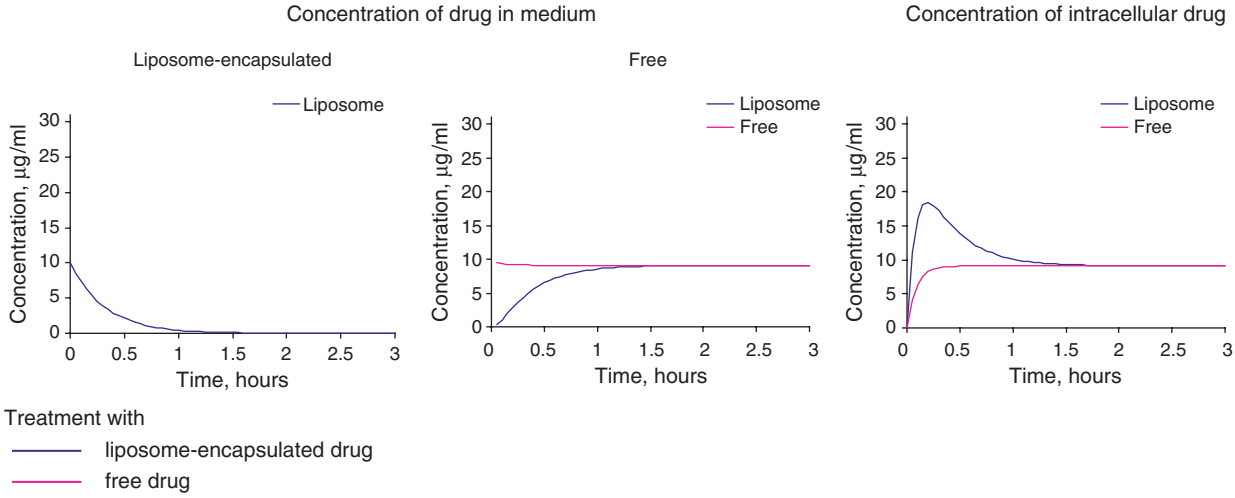


Fig. 4. Cell treatment with liposome-encapsulated and free drug. Initial drug concentration in medium was 10 μg/ml both for liposome-encapsulated and free drug treatment; cell concentration was 10^7 cells/ml.

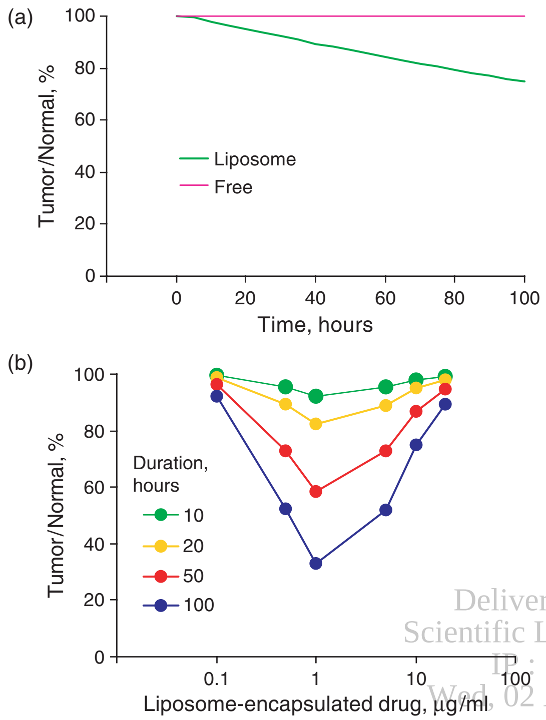


Fig. 5. Simulation of treatment of mixture of normal and tumor cells: (a) For liposome delivery and free toxin; (b) for different liposome encapsulated toxin concentrations (0.1, 0.5, 1, 5, 10, and 20 μg/ml) and different treatment durations (10, 20, 50, and 100 hours). Initial ratio of cell concentrations (tumor to normal) was 1:1 and it was 10^7 cell/ml normal and tumor cells in medium.

shown in the Table II were equal for normal and tumor cells. As shown in Figure 5a, drug delivery with liposome changes proportion of tumor to normal cells with time. After 100 hours of treatment the ratio tumor/normal cells is about 70% in mixture. Figure 5b demonstrates the nonlinear dependence of tumor to normal ratio on liposome concentration. For low concentration there is no effect of treatment and the ratio is about 100% for all treatment durations, for high liposome-encapsulated drug concentration the ratio is also about 100%. As it is shown in figure there is an optimal value for liposome-entrapped drug concentration (about 1 μg/ml) and the effect of treatment for this concentration increases with the increase of treatment duration.

5. DISCUSSION

In this study kinetic model of delivery of free and liposome-encapsulated drug to the target cells was developed. The model consists of 4 differential equations and describes accumulated intracellular amount of drug per target cells, concentration of free and liposome-encapsulated drug in medium, and concentration of target cells.

Liposome-encapsulated or free drug or toxin molecules can be delivered to cell cytosol either by binding of liposomes to cell surface and subsequent release of encapsulated drug by fusion or internalization or by transport of free molecules through cell membrane. We used only one parameter which is responsible for the rate of delivery: k_L for liposome-entrapped and k_F for free molecules, respectively. Active or passive transport of drug molecules out of cells increases free but not the liposome-encapsulated drug concentration in medium. For passive transport we assumed $k_{F-} = k_{F+}$, so active transport inside and outside cell can be modeled with inequalities $k_{F-} < k_{F+}$ or $k_{F-} > k_{F+}$.

Model solution were fitted to the experimental data published in Ref. [23]. First, values of parameters responsible for the toxin delivery into cells (k_L and k_F) were estimated using the data about the uptake of DOX into B16F10 cells after a 3-hour exposure. These constants can be easily estimated with the formula: (Intracellular DOX amount)/(Extracellular DOX concentration)/(Time of exposure) assuming that there was no changes in extracellular concentration and there is no efflux of free DOX from the cell. As it was shown in Ref. [23] in presence of 0.5–20 μM of DOX in medium uptake of DOX was about femtomoles per cell (from 0.6 to 32 fmole/cell) after 3 hours of exposure, so one can calculate values of about 3×10^{-7} and 1×10^{-7} ml $\cdot$ cell^{-1} $\cdot$ hour^{-1} for k_L and k_F, respectively. Amount of 10 femtomoles of DOX per cell corresponds to about 538 μg/ml (molecular weight of DOX is 580 and cell volume is about 10^{-8} ml). These relatively high values can be explained by binding of the drug molecules to lipids and nucleic acids inside the cell (Szoka, personal communication) which can decrease intracellular concentration of unbound DOX and concentration of DOX bound to DNA. These data demonstrates linear dependence of intracellular DOX at 3 hours on extracellular concentration of drug for liposome delivery. For free DOX delivery dose-response curve is different: Linear dependence was shown only for low concentrations of extracellular DOX (0.5–10 μg/ml) with saturation at 20 μg/ml.

Parameter responsible for the efflux of drug from treated cells can be estimated using the data on cellular uptake of DOX by non-small cell lung tumor cells.[32] These data represent dose-response curves for different time of exposure (0.5–3 hours) of different concentrations of DOX (0.2–10 μg/ml) to cells. The data demonstrates initial saturation (for extracellular concentrations up to 2 μg/ml) followed by linear increase in uptake up to 10 μg/ml. Separate fitting of each dynamics for different DOX concentrations showed the following ranges of the rates of influx and efflux: $k_{F+} = (0.9\text{–}5) \times 10^{-6}$ ml $\cdot$ cell^{-1} $\cdot$ hour^{-1} and $k_{F-} = (5\text{–}6) \times 10^{-8}$ ml $\cdot$ cell^{-1} $\cdot$ hour^{-1}. So, the rate of efflux of DOX from cells is relatively low and can be neglected.

It was assumed that liposome and free drug molecules are stable inside and outside cells so only three parameters describe each dose-response curve shown in Figure 3. Curves corresponding to the different time of exposure (immediate treatment) or exposure and subsequent delay before measuring survivals (delayed treatment) were fitted separately with fixed parameters of DOX delivery and

released parameters of cell kill due to the presence of intracellular DOX (d_I, K, and m). Results of fitting show that for all protocols model solution is applicable for simulating the experimental data (Fig. 3, parameter values are shown in Table II). Average values of d_I and m parameters are about 0.05 hour^{-1} and 1.5 with exception for immediate 24 hours treatment for liposome and free toxin delivery. For these treatment the maximum increase in cell death rate was relatively high (about 0.16 hour^{-1}) and value of slope parameter was about 4. It means that increase in DOX concentration during treatment provides sharp effect on cell killing for 24-hours treatment for free and liposome-entrapped toxin. We can not see this effect for delayed 24-hours treatment because of the saturation effect: Cell killing curves for these protocols are almost the same for 48, 72, and 96 hours of delayed treatments.

Model dynamics for treatment with liposome-encapsulated and free toxin molecules delivery is shown in Figure 4. For this simulation $k_L > k_F$, so the total amount of intracellular toxin per cell increases faster for delivery with liposomes but this increase is transient if there is an efflux of drug from the cells. The existence of efflux from the cells leads to the equilibration of intracellular and extracellular drug concentrations, and the rate of this equilibration depends on the values of parameters k_{F-} and k_{F+}. Increase in cell concentration can change significantly system dynamics: Interval of time corresponding to increased intracellular toxin concentration shortens and for high concentration of cells dynamics of intracellular drug for delivery of free and liposome-entrapped drug will be close.

Figure 5 shows the results of calculation when mixture of two types of cells (tumor and normal) was treated with liposome-encapsulated drug. It was assumed that liposome can deliver toxin more effectively (2 time faster) comparing to free toxin. Although concentration of both types of cells decreased with time due to delivery of toxin into cell and increased cells death, there is no change with time for the ratio tumor to normal cells for free toxin delivery (Fig. 5a). For liposome delivery this ratio decreased from 100% at the beginning up to 75% after 100 hours of treatment. This effect depends nonlinearly on of liposome concentration (Fig. 5b). These results show that there is an optimal concentration of liposome-encapsulated drug giving maximal effect of treatment for mixture of tumor and normal cells.

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